

## ANGIOGENIC PEPTIDES AND USES THEREOF

FIELD AND BACKGROUND OF THE INVENTION

5           The present invention relates to peptides that are capable of promoting angiogenesis and to the use thereof in the treatment of angiogenesis-dependent diseases, such as ischemic vascular diseases.

          Angiogenesis is the process of generating new capillary blood vessels and involves an interplay between cells and soluble factors (1). In brief, activated  
10   endothelial cells migrate and proliferate to form new vessels, which are surrounded by layers of periendothelial cells; small blood vessels are surrounded by pericytes and large blood vessels are surrounded by smooth muscle cells.

          Numerous factors regulate the angiogenic process. These include soluble factors and tissue oxygen. In the past two decades, a number of angiogenic molecules  
15   which positively regulate the angiogenic process were elucidated. These include Vascular Endothelium Growth Factor (VEGF), basic Fibroblast Growth Factor (bFGF), acidic FGF/FGF-1, hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), and others (2). As mentioned, oxygen conditions also have important implications for the physiological and pathological angiogenic process (3). Under hypoxic conditions,  
20   VEGF gene expression is induced both in endothelial cells and pericytes to produce secretory forms of VEGF. VEGF, in turn, may bind to VEGF receptor-2 (Kdr) or VEGF receptor-1 (VEGFR-1; Flt-1) expressed on endothelial cells in an autocrine or paracrine manner, thereby causing proliferation of endothelial cells, which may lead to angiogenesis. Basal amounts of vascular VEGF synthesized under normoxia  
25   promote the maintenance of microvascular homeostasis (5). Expression of VEGF receptor 1 mRNA (Flt-1) was found to be up-regulated in peri-ischemic endothelial cells and in the infarcted core of endothelial cells and periphery, with peak expression of VEGFR-1 in endothelial cells. Gene expression of VEGFR-1 is directly inducible by hypoxia, as in the case of VEGF. Twenty-four hours following hypoxia-induced  
30   VEGF gene expression, concurrent with the expression of the VEGFR-1 and 2 (Kdr) genes, endothelial cells begin to proliferate (6, 7).

          Hypoxia-inducible gene products that participate in these cellular responses include erythropoietin, VEGF, and glycolytic enzymes (8). Hypoxia can directly

enhance the expression of bFGF mRNA in pericytes. Increased expression of bFGF may play an important role in pericyte proliferation and in differentiation of pericytes and smooth muscle cells (9).

Angiogenesis-dependent diseases result when the angiogenic process is  
5   disregulated, resulting in excessive amounts of new blood vessels or an insufficient  
number of blood vessels. Insufficient angiogenesis is related to a large number of  
diseases and conditions, such as coronary artery diseases and delayed wound healing.  
To date, cardiovascular diseases are the leading cause of mortality in the United  
States, Europe, and Israel. In the United States, approximately one million deaths per  
10   year are attributed to cardiac causes, fifty percent of which are attributed to Coronary  
Artery Disease (CAD). The major morbidity from CAD is a result of obstructive  
coronary artery narrowing and the resultant myocardial ischemia. CAD affects more  
than 13 million people, and its annual economic burden is in excess of sixty billion  
U.S. Dollars.

15       Mechanical revascularization of obstructive coronary stenoses by  
percutaneous techniques, including percutaneous transluminal angioplasty and stent  
implantation, is used to restore normal coronary artery blood flow. In addition,  
coronary artery occlusion bypass surgery is performed using arterial and venous  
conduits as grafts onto the coronary arterial tree. These treatment modalities have  
20   significant limitations in individuals with diffuse atherosclerotic disease or severe  
small vessel coronary artery disease, in diabetic patients, as well as in individuals who  
have already undergone surgical or percutaneous procedures.

For these reasons, therapeutic angiogenesis, aimed at stimulating new blood  
vessel growth, is highly desirable. The therapeutic concept of angiogenesis therapy is  
25   based on the premise that the existing potential for vascular growth inherent to  
vascular tissue can be utilized to promote the development of new blood vessels under  
the influence of the appropriate angiogenic molecules.

Therapeutic angiogenesis defines the intervention used to treat local  
hypovascularity by stimulating or inducing neovascularization for the treatment of  
30   ischemic vascular disease.

Animal studies have proven the feasibility of enhancing collateral perfusion  
and function via angiogenic compounds. Those experiments proved that exogenous  
administration of angiogenic growth factors or their genetic constructs could promote

collateral vessel growth in experimental models of chronic ischemia. Although such studies demonstrated proof of concept, additional studies raise issues that still have not been resolved, such as the duration of exposure of the vessels to angiogenic factors and the brief half-lives of such proteins (10).

5        Synthetic peptides encompassing portions of proteins have become supportive tools for understanding the molecular mechanisms associated with protein biological functions. The use of short peptides constructed from specific regions of human FGF and VEGF that have the potential to efficiently agonize or antagonize the biological functions of the growth factor family members has been described (11). Several  
10       groups have reported the use of intact cells to screen a phage display peptide library to identify cell surface-binding peptides (12). A peptide-based ligand receptor map of the VEGF family was constructed by screening human endothelial cells stimulated with VEGF with a peptide library (13). Another study has described the screening of a 12-mer phage display peptide library on VEGF-2 receptor protein (14).

15       While reducing the present invention to practice, the present inventors used a 12-mer phage display peptide library to uncover peptides which are able to bind the cell-surface of endothelial cells incubated under normoxic or hypoxic conditions. Such peptides were shown to trigger angiogenic processes including endothelial cell-proliferation and vascularization. As such, these peptides can be used to treat various  
20       angiogenesis-dependent diseases, such as ischemic vascular diseases. Furthermore, characterization of the nature of endothelial cell signaling by these peptides will provide the basis for the development of targeted angiogenic therapy for morbidities, such as cardiovascular disease.

## 25       SUMMARY OF THE INVENTION

      According to one aspect of the present invention there is provided peptide selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12.

      According to another aspect of the present invention there is provided a peptide comprising an amino acid sequence selected from the group consisting of  
30       SEQ ID NOs: 2, 4, 6, 8, 10, and 12, the peptide being no more than 50 amino acid residues in length.

According to yet another aspect of the present invention there is provided a peptide comprising an amino acid sequence as set forth in SEQ ID NO:13, 27, or 32, the peptide being at least 6 and no more than 50 amino acid residues in length.

5 According to still another aspect of the present invention there is provided a composition-of-matter comprising at least two peptides, each independently selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12.

10 According to an additional aspect of the present invention there is provided a pharmaceutical composition comprising a therapeutically effective amount of a peptide having an amino acid sequence as set forth in SEQ ID NO:13, 27, or 32, the peptide being at least 6 and no more than 50 amino acid residues in length, and a pharmaceutically acceptable carrier or diluent.

15 According to yet an additional aspect of the present invention there is provided a pharmaceutical composition comprising a therapeutically effective amount of a peptide selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12 and a pharmaceutically acceptable carrier or diluent.

20 According to still an additional aspect of the present invention there is provided a pharmaceutical composition comprising a therapeutically effective amount of a peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12, the peptide being no more than 50 amino acid residues in length, and a pharmaceutically acceptable carrier or diluent.

25 According to a further aspect of the present invention there is provided a method of promoting angiogenesis in a tissue of a subject, the method comprising providing to the subject a therapeutically effective amount of a peptide having an amino acid sequence as set forth in SEQ ID NO:13, 27, 32 the peptide being at least 6 and no more than 50 amino acid residues in length, to thereby promote angiogenesis in the subject.

According to further features in preferred embodiments of the invention described below, the peptide is selected from the group consisting of SEQ ID NOs:2, 6, and 12.

30 According to still further features in the described preferred embodiments the amino acid sequence is selected from the group consisting of SEQ ID NOs:2, 6, and 12.

According to still further features in the described preferred embodiments the peptide is a linear peptide or a cyclic peptide.

According to yet a further aspect of the present invention there is provided a method of promoting angiogenesis in a tissue of a subject, the method comprising providing to the subject a therapeutically effective amount of a peptide selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12, to thereby promote angiogenesis in the subject.

According to still a further aspect of the present invention there is provided a method of promoting angiogenesis in a tissue of a subject, the method comprising providing to the subject a therapeutically effective amount of a peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12, the peptide being no more than 50 amino acid residues in length, to thereby promote angiogenesis in the subject.

According to still further features in the described preferred embodiments the subject suffers from arteriosclerosis, retinopathy, remodeling disorder, von Hippel-Lindau syndrome, diabetes, and/or hereditary hemorrhagic telangiectasia.

According to still a further aspect of the present invention there is provided a nucleic acid construct comprising a polynucleotide sequence encoding the peptide of the present invention.

According to still further features in the described preferred embodiments the nucleic acid construct further comprises a promoter.

According to still a further aspect of the present invention there is provided a composition for targeting a drug to endothelial cells, the composition comprising the drug fused to a peptide having an amino acid sequence as set forth in SEQ ID NO:13, 27, or 32, the peptide being at least 6 and no more than 50 amino acid residues in length.

According to still further features in the described preferred embodiments the drug is selected from the group consisting of a toxin, a chemotherapeutic agent, and a radioisotope.

According to still a further aspect of the present invention there is provided a composition for targeting a drug to endothelial cells, the composition comprising the drug fused to a peptide selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12.

According to still a further aspect of the present invention there is provided a composition for targeting a drug to endothelial cells, the composition comprising the drug fused to a peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12, the peptide being no more than 50 amino acid residues in length.

According to still a further aspect of the present invention there is provided a method of identifying putative angiogenic molecules, the method comprising: (a) providing endothelial cells having peptides bound thereto, each of the peptides having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, and 12, the peptide being no more than 50 amino acid residues in length; and (b) identifying a molecule capable of displacing the peptides from the endothelial cells to thereby identify putative angiogenic molecules.

The present invention successfully addresses the shortcomings of the presently known configurations by providing peptides which are capable of promoting angiogenesis and as such can be used to treat angiogenesis-dependent diseases, such as ischemic vascular diseases.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

## BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the

description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1a-b are bar graphs depicting the binding of peptide-presenting phages at a concentration of  $10^9$  (Figure 1a) or  $10^{10}$  (Figure 1b) phage per well, to ECs under normoxic conditions and following 3, 6, and 24 hours of hypoxia. The bars represent the binding to ECs of 15 different peptide-presenting phage (VL, LP, TR, ST, QF, NS, SP, YR, LT, HR, HY, SV, TP, NR, and SA) and the control (NO, unmodified M13 phage) following a 2-hour incubation. Absorbance at 450 nm, produced by anti-M13 HRP antibody, which detects peptide-presenting phages attached to ECs in the presence of tetramethyl benzidine liquid substrate, was measured using an ELISA reader.

FIG. 2 is a bar graph depicting the effect of peptide-presenting phages on ECs proliferation. Six peptide-presenting phages (VL, TR, YR, QF, LT, SP) each at a concentration of  $10^6$  were incubated with ECs in serum free media for 24 hours. Data was obtained by measuring radioactive [ $^3\text{H}$ ]-Thymidine uptake into ECs (cpm/min) in the last 6 hours of incubation, and presented as a percent above ECs proliferation induced by control phages (NO, unmodified M13 phages).

FIGs. 3a-b are bar graphs depicting the effect of direct activation of peptide-presenting phages on ECs migration. The migration of ECs was assayed in the presence of  $10^5$  (Figure 3a) or  $10^6$  (Figure 3b) peptide-presenting phages per well and was compared to negative (ECs, or ECs in the presence of NO phages - unmodified M13 phages) or positive (the angiogenic molecule- VEGF) control. The bars compare ECs migration induced by 12 peptide-presenting phages (VL, LP, QF, SP, TR, NS, SV, LA, LT, YR, TP and SA) following 5 hours of incubation in migration chambers. Data was obtained by measuring the fluorescent enhancement of the CyQuant GR dye molecular probe bound to cellular nucleic acid of lysed migratory cells using the Fluorescent ELISA reader at 480/520 nm and is expressed as Relative Fluorescence Units (RFU).

FIGs. 4a-b are bar graphs depicting chemo-attraction of ECs by peptide-presenting phages added to the feeder tray of the migration chamber. Peptide-presenting phages were used at a concentration of  $10^5$  (Figure 4a) or  $10^6$  (Figure 4b) phages per well and were compared to negative controls (ECs or ECs in the presence

of NO phages - unmodified M13 phages). The bars compare ECs chemo-attraction induced by 12 peptide-presenting phages (VL, LP, QF, SP, TR, NS, SV, LA, LT, YR, TP and SA) following 5 hours of incubation in migration chamber. Data was obtained and presented as described for Figures 3a-b, hereinabove.

5        FIG. 5 is a bar graph depicting the proliferation of ECs in arterial rings in the presence of peptide-presenting phages ( $10^6$ ) as compared with negative control (NO phage, unmodified M13 phages) and positive control (the angiogenic molecule-FGF). The bars compare ECs proliferation induced by six peptide-presenting phages (SP, LT, YR, TR, VL, and QF) following 7 days incubation in DMEM containing 10 % FCS  
10        (37 °C with 5 % CO<sub>2</sub>). Data was obtained by an XTT assay (O.D. 450 nm).

FIGs. 6a-i are graphs depicting the specific binding of synthetic peptides to Peripheral Blood Lymphocytes (PBL, Figure 6a) or ECs (Figure 6b-i). The graphs represent flow cytometry analysis of 100,000 cells incubated for 2 hours with 4 or 6  $\mu$ g of synthetic peptides. Figures 6a-b are the results of flow cytometry analysis  
15        presenting the gates chosen either for (Figure 6a) peripheral blood lymphocytes or for (Figure 6b) endothelial cells analysis. The dots represent the dispersion of FITC labeled cells, according to their size (horizontal axis) versus their granulation (vertical axis). Figures 6c-i are the results of flow cytometry analysis presenting the percent binding to ECs and mean fluorescence of synthetic peptides: Figure 6c - no peptide;  
20        Figure 6d – SP; Figure 6e – QF; Figure 6f – LT; Figure 6g – YR; Figure 6h – TR; Figure 6i - VL. The graphs represent flow cytometry analysis of  $10^6$  ECs incubated for 2 hours with 4  $\mu$ g of synthetic peptides (green line), 6  $\mu$ g of synthetic peptides (red line), or isotype control (black line).

FIG. 7 is a bar graph demonstrating binding of synthetic peptides to PBL and  
25        ECs. The graph represents flow cytometry analysis of 5  $\mu$ g FITC labeled synthetic peptide (SP, YR, LT, VL, QF and TR) bound to 100,000 PBLs and ECs. The bars compare mean fluorescence (emitted by the labeled synthetic peptides following 2 hours incubation with PBLs or ECs. Data was collected using FACS.

FIGs. 8a-b are graphs depicting the effect of synthetic peptides on cells  
30        proliferation. Figure 8a illustrates the proliferation of ECs induced by LP, ST, TR, and VL at concentrations of 0.05, 0.1, 1, 10, and 100 ng/ml, following 24 hours incubation in EBM-2. Figure 8b illustrates the proliferation of MVECs induced by

LT SP, or YR at concentrations of 0.1, 1, 10, and 100 ng/ml following 24 hours incubation in EBM-MV. Results are expressed as [<sup>3</sup>H]-Thymidine uptake by cells incubated with peptides minus control (cells incubated in EBM-2 and EBM-MV, respectively). Data was obtained by measuring radioactive [<sup>3</sup>H]-Thymidine uptake into cells in a scintillation β counter by cpm/min in the last 6 hours of incubation.

FIGs. 9a-c are graphs depicting the effect of the synthetic peptides on ECs migration. Figure 9a-c are graphs illustrating the migration of ECs induced by LT (Figure 9a), SP (Figure 9b), or VL and TR (Figure 9c) at concentrations of 5, 10, 20 and 50 ng/ml following 5 hours of incubation in migration chamber. Data was obtained and presented as described for Figures 3a-b, hereinabove.

FIG. 10 is a graph depicting the time dependent effect of peptide incubation on ECs migration. The figure presents the migration of ECs as induced by 1 ng/ml synthetic peptide (LT, QF, SP, TR, VL and YR) following 5 and 15 hours of incubation in migration chamber. Data was obtained and presented as described for Figures 3a-b, hereinabove.

FIGs. 11a-b are graphs depicting effect of the synthetic peptides on MVECs migration. The graphs are illustrating the migration of MVECs (Figure 11a) and migration activation of MVECs (Figure 11b) induced by LT, SP, YR, TR, VL, QF and FGF at concentrations of 1 and 10 ng/ml, following 5 hours of incubation in migration chamber. Data was obtained and presented as described for Figures 3a-b, hereinabove.

FIG. 12 is a graph depicting the effect of synthetic peptides on arterial ring sprouting. The graph presents the proliferation of ECs in arterial rings induced by four synthetic peptides (*i.e.*, QF, YR, LT and VL) at concentrations 1, 10, 100 and 1000 ng/ml following 7 days incubation in DMEM containing 10 % FCS (37 °C with 5 % CO<sub>2</sub>). Data was obtained by estimation of cell proliferation by an XTT assay (O.D. 450 nm).

FIGs. 13a-j are photomicrographs depicting the effect of the peptides on cells tube formation. Figures 13a-e demonstrate MVEC tube formation induced by 8 hours incubation of VEGF (Figure 13b), YR (Figure 13c), QF (Figure 13d), VL (Figure 13e), as compared to untreated control (Figure 13a). Photos were taken after 8 incubation; Magnification x 100. Figures 13f-j demonstrate EC tube formation

induced by 20 hours incubation of FGF (Figure 13g), YR (Figure 13h), QF (Figure 13i), VL (Figure 13j), as compared to untreated control (Figure 13f).

FIGs. 14a-e are bar graphs depicting the effect of synthetic peptides on gene expression of the following genes in MVECs: Figure 14a - VEGF-A; Figure 14b - VEGF-C; Figure 14c - FLT-1; Figure 14d - KDR; Figure 14e - HIF-1 $\alpha$ . The synthetic peptides (LT, QF, SP, TR, YR and VL at concentration of 1 ng/ml) or VEGF (at concentration of 10 ng/ml) were added to the cells and gene expression was determined using real-time PCR 1.5 and 6 hours following the peptide or VEGF addition. Results are presented as net expression ratio of treated cells as compared to untreated controls.

FIG. 15 is a bar graph demonstrating the intensity of synthetic peptides binding to ECs exposed to the effect of hypoxia treatment. Flow cytometry analysis was effected on the binding of 6  $\mu$ g FITC labeled synthetic peptide (LT, QF, SP, TR, VL and YR) to 10<sup>5</sup> untreated ECs or ECs after hypoxia. The bars compare mean fluorescence (488 nm) obtained after 2 hours incubation of the FITC labeled synthetic peptides with ECs. Data was collected using FACS.

FIGs. 16a-b are graphs depicting the intensity of synthetic peptides binding to ECs exposed to the effect of hypoxia. The graphs present flow cytometry analysis of 6  $\mu$ g FITC labeled SP peptide (Figure 16a) or LT peptide (Figure 16b) to 10<sup>5</sup> ECs following 2 hours incubation. Red line – ECs without hypoxia; green line - ECs after hypoxia; black line - isotype control.

FIGs. 17a-f are graphs depicting the effect of synthetic peptides on cells proliferation. Figures 17a-c illustrate the proliferation of HUVECs induced by LT (Figure 17a), SP (Figure 17b), or QF (Figure 17c) at concentrations of 0.01, 1, 10, and 100 ng/ml, following 24 hours incubation in EBM-2. Figures 17d-f illustrate the proliferation of MVECs induced by LT (Figure 17d), SP (Figure 17e), or QF (Figure 17f) at concentrations of 0.01, 1, 10, and 100 ng/ml following 24 hours incubation in EBM-MV. The plots compare the proliferation of cells under normal conditions (control), under hypoxic conditions or after hypoxic conditions. Data was obtained by measuring radioactive [<sup>3</sup>H]-Thymidine uptake into cells in a scintillation  $\beta$  counter by cpm/min in the last 6 hours of incubation.

FIGs. 18a-e are photomicrographs depicting the effect of SP on HUVEC (Figures 18a-c) and MVEC (Figures 18d-e) tube formation under hypoxic conditions. Figures 18a-c demonstrate EC tube formation induced by 18 hours incubation with FGF (Figure 18b), or SP (Figure 18c) as compared to control (Figure 18a). Figures 18d-e demonstrate MVEC tube formation induced by 18 hours incubation with FGF (Figure 18d), or SP (Figure 18e). Photos were taken after 18 hours incubation. Magnification x 100.

FIGs. 19a-e are photomicrographs depicting the effect of synthetic peptides on vascularization of mouse ears. The figures demonstrate the vascularization as induced by subcutaneous injection of VEGF (100 ng/mouse ear; Figure 19a), LT (10 µg/ear; Figure 19b), YR (10 µg/ear; Figure 19c), QF (µg/ear; Figure 19d), or SP (Figure 19e, 0.1 µg/ear). Each injection was carried out in a final volume of 10 µl, *i.e.*, 10 µg/10 µl PBS, 0.1 µg/10 µl PBS or 10 µl PBS (Control). Photos were taken 2 days following injection.

FIGs. 20a-b are photomicrographs depicting the effect of TR peptide on vascularization of mouse ear. Shown are histology sections demonstrating vascularization and neo-vascularization induced by 10 µg TR (Figure 20b) as compared to control (Figure 20a). Note, injection of TR peptide reveals large blood vessel formation and neovascularization as demonstrated by capillary blood vessels with single erythrocyte cell.

FIG. 21 is a bar graph depicting median flux of ischemic hind limb/control in a rat ischemic hind limb model. The Figure presents the ischemic hind limb blood flow measured at days 4, 7, 9 and 13. Results are the mean of 4, 7, 9, and 13 days after peptide inoculation after 600 µg of VL, LT, QF, TR, SP, YR, FGF or PBS injection to the ischemic leg as compared to the other leg. Results are expressed as OP/control x100 median flux (Percent median flux of the operated leg versus non operated control leg). Data was obtained at days 4, 7, 9, 13 using a Laser Doppler Blood Flow analyzer.

FIGs. 22a-c illustrate the uncovering a conserved sequence motif which is shared by the peptides of the present invention and the mouse VEGF-B (Swiss-Prot Accession: VEGF\_MOUSE). Figure 22a – the amino acid sequences of the peptides of the present invention; Figure 22b - alignment of the amino acid sequences of VL,

QF, YR and TR and scanning by e-Motif uncovers a conserved motif “pw[il][de].y”; Figure 22c – alignment of the amino acid sequences of VL, QF, YR and TR with mouse VEGF-B.

## 5 DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of peptides, which can be used for promoting tissue angiogenesis. Specifically, the present invention can be used to treat angiogenesis-dependent diseases, such as ischemic vascular diseases.

10 The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is 15 to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Angiogenesis is the process of generating new capillary blood vessels involving an interplay between cells and soluble factors (1). The process is characterized by the migration of activated endothelial cells that proliferate to form 20 new vessels, which are surrounded by layers of periendothelial cells, including pericytes for small blood vessels and smooth muscle cells for large blood vessels.

Angiogenesis-dependent diseases are a consequence of an imbalanced angiogenic process resulting in an excessive amount of new blood vessels or insufficient number of blood vessels. Insufficient angiogenesis is related to a large 25 number of diseases and conditions, such as coronary artery diseases and delayed wound healing.

Therapeutic angiogenesis is aimed at stimulating new blood vessel growth. The concept of such a therapy is based on the premise that the inherent potential of vascularization in a vascular tissue can be utilized to promote the development of new 30 blood vessels under the influence of the appropriate angiogenic molecules.

While reducing the present invention to practice, the present inventors used a 12-mer phage display peptide library to uncover peptides that are able to bind the cell-surface of endothelial cells incubated under normoxic or hypoxic conditions.

As is illustrated in the Examples section which follows, the peptides of the present invention triggered angiogenic reactions including, endothelial cell-proliferation and migration, aortic ring sprouting, tube formation and *in-vivo* vascularization. These findings suggest that the peptides of the present invention can be used in the treatment of various angiogenesis-dependent diseases, such as ischemic-vascular diseases. Furthermore, characterization of the nature of endothelial cell signaling by these peptides will provide the basis for the development of targeted angiogenic therapy for diseases, such as cardiovascular disease.

Thus, according to one aspect of the present invention there is provided a peptide including an amino acid sequence as set forth in SEQ ID NO: 2, 4, 6, 8, 10 or 12, the peptide is at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, and no more than 50 amino acid residues in length.

According to another aspect of the present invention there is provided a peptide including an amino acid sequence selected from the group consisting of SEQ ID NOs: NO:13, 27 or 32, the peptide is at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, and no more than 50 amino acid residues in length.

As is shown in Example 6 of the Examples section, the peptides of this aspect of the present invention share a conserved amino acid sequence (SEQ ID NO:13, 27 or 32) with mammalian vascular endothelial growth factor B (VEGF-B, SwissProt/TrEMBL Accession: VEGF\_MOUSE), thereby substantiating the angiogenic function attributed to the peptides of this aspect of the present invention.

Preferably, the peptide of the present invention includes the sequence set forth by SEQ ID NO: 2, 6, 8 or 12, more preferably the peptide of the present invention includes the sequence set forth by SEQ ID NO:6 or 8.

According to another preferred embodiment of this aspect of the present invention the amino acid sequence is as set forth in SEQ ID NO: 2, 6, 8, or 12, preferably the amino acid sequence is as set forth in SEQ ID NO:6 or 8.

The present invention also envisages the use of peptides containing more than one consensus sequence as provided in SEQ ID NO:14.

According to yet another aspect of the present invention there is provided a peptide including an amino acid sequence as set forth in SEQ ID NO: 14, the peptide is at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, and no more than 50 amino acid residues in length.

The term "peptide" as used herein encompasses native peptides (either degradation products, synthetically synthesized peptides or recombinant peptides) and peptidomimetics (typically, synthetically synthesized peptides), as well as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications rendering the peptides more stable while in a body or more capable of penetrating into cells. Such modifications include, but are not limited to N terminus modification, C terminus modification, peptide bond modification, including, but not limited to, CH<sub>2</sub>-NH, CH<sub>2</sub>-S, CH<sub>2</sub>-S=O, O=C-NH, CH<sub>2</sub>-O, CH<sub>2</sub>-CH<sub>2</sub>, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein.

Further details in this respect are provided hereinunder. Peptide bonds (-CO-NH-) within the peptide may be substituted, for example, by N-methylated bonds (-N(CH<sub>3</sub>)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-), ketomethylen bonds (-CO-

CH<sub>2</sub>-),  $\alpha$ -aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba  
bonds (-CH<sub>2</sub>-NH-), hydroxyethylene bonds (-CH(OH)-CH<sub>2</sub>-), thioamide bonds (-CS-  
NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), peptide  
derivatives (-N(R)-CH<sub>2</sub>-CO-), wherein R is the "normal" side chain, naturally  
presented on the carbon atom.

These modifications can occur at any of the bonds along the peptide chain and  
even at several (2-3) at the same time.

Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for  
synthetic non-natural acid, such as Phenylglycine, TIC, naphthylelanine (Nol), ring-  
methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

In addition to the above, the peptides of the present invention may also include  
one or more modified amino acids or one or more non-amino acid monomers (e.g.  
fatty acids, complex carbohydrates etc).

As used herein in the specification and in the claims section below the term  
"amino acid" or "amino acids" is understood to include the 20 naturally occurring  
amino acids; those amino acids often modified post-translationally *in vivo*, including,  
for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual  
amino acids including, but not limited to, 2-amino adipic acid, hydroxylysine,  
isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino  
acid" includes both D- and L-amino acids.

Tables 1 and 2 below list naturally occurring amino acids (Table 1) and non-  
conventional or modified amino acids (e.g., synthetic, Table 2) which can be used  
with the present invention.

**Table 1**

<i>Amino Acid</i>	<i>Three-Letter Abbreviation</i>	<i>One-letter Symbol</i>
alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
glycine	Gly	G
Histidine	His	H
isoleucine	Ile	I
leucine	Leu	L

Lysine	Lys	K
Methionine	Met	M
phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
Valine	Val	V
Any amino acid as above	Xaa	X

Table 2

<i>Non-conventional amino acid</i>	<i>Code</i>	<i>Non-conventional amino acid</i>	<i>Code</i>
$\alpha$ -aminobutyric acid	Abu	L-N-methylalanine	Nmala
$\alpha$ -amino- $\alpha$ -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane-	Cpro	L-N-methylasparagine	Nmasn
carboxylate		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-	Norb	L-N-methylglutamine	Nmgin
carboxylate		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisoleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Das	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	$\alpha$ -methyl-aminoisobutyrate	Maib
D-valine	Dval	$\alpha$ -methyl- $\gamma$ -aminobutyrate	Mgab
D- $\alpha$ -methylalanine	Dmala	$\alpha$ -methylcyclohexylalanine	Mchexa
D- $\alpha$ -methylarginine	Dmarg	$\alpha$ -methylcyclopentylalanine	Mcpen
D- $\alpha$ -methylasparagine	Dmasn	$\alpha$ -methyl- $\alpha$ -naphthylalanine	Manap
D- $\alpha$ -methylaspartate	Dmasp	$\alpha$ -methylpenicillamine	Mpen
D- $\alpha$ -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D- $\alpha$ -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D- $\alpha$ -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D- $\alpha$ -methylisoleucine	Dmile	N- amino- $\alpha$ -methylbutyrate	Nmaabu
D- $\alpha$ -methylleucine	Dmleu	$\alpha$ -naphthylalanine	Anap
D- $\alpha$ -methyllysine	Dmlys	N-benzylglycine	Nphe

D- $\alpha$ -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D- $\alpha$ -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D- $\alpha$ -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D- $\alpha$ -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D- $\alpha$ -methylserine	Dmser	N-cyclobutylglycine	Ncbut
D- $\alpha$ -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D- $\alpha$ -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D- $\alpha$ -methyltyrosine	Dmtty	N-cyclodecylglycine	Ncdec
D- $\alpha$ -methylvaline	Dmval	N-cyclododecylglycine	Ncdod
D- $\alpha$ -methylalnine	Dnmala	N-cyclooctylglycine	Ncoct
D- $\alpha$ -methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D- $\alpha$ -methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D- $\alpha$ -methylasparatate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D- $\alpha$ -methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- $\gamma$ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmtet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nva
D-N-methyltyrosine	Dnmtyr	N-methyl- $\alpha$ -naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
$\gamma$ -aminobutyric acid	Gabu	N-( <i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- $\alpha$ -methylalanine	Mala
L- $\alpha$ -methylarginine	Marg	L- $\alpha$ -methylasparagine	Masn
L- $\alpha$ -methylaspartate	Masp	L- $\alpha$ -methyl- <i>t</i> -butylglycine	Mtbug
L- $\alpha$ -methylcysteine	Mcys	L-methylethylglycine	Metg
L- $\alpha$ -methylglutamine	Mgln	L- $\alpha$ -methylglutamate	Mglu
L- $\alpha$ -methylhistidine	Mhis	L- $\alpha$ -methylhomo phenylalanine	Mhphe
L- $\alpha$ -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolethyl)glycine	Nhis
D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl- $\gamma$ -aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmtet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval

D-N-methyltyrosine	Dnmtyr	N-methyl-naphthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
$\gamma$ -aminobutyric acid	Gabu	N-( <i>p</i> -hydroxyphenyl)glycine	Nhtyr
L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L- $\alpha$ -methylalanine	Mala
L- $\alpha$ -methylarginine	Marg	L- $\alpha$ -methylassparagine	Masn
L- $\alpha$ -methylasspartate	Masp	L- $\alpha$ -methyl- <i>t</i> -butylglycine	Mtbug
L- $\alpha$ -methylcysteine	Mcys	L-methylethylglycine	Metg
L- $\alpha$ -methylglutamine	Mgln	L- $\alpha$ -methylglutamate	Mglu
L- $\alpha$ -methylhistidine	Mhis	L- $\alpha$ -methylhomophenylalanine	Mhphe
L- $\alpha$ -methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L- $\alpha$ -methylleucine	Mleu	L- $\alpha$ -methyllysine	Mlys
L- $\alpha$ -methylmethionine	Mmet	L- $\alpha$ -methylnorleucine	Mnle
L- $\alpha$ -methylnorvaline	Mnva	L- $\alpha$ -methylornithine	Morn
L- $\alpha$ -methylphenylalanine	Mphe	L- $\alpha$ -methylproline	Mpro
L- $\alpha$ -methylserine	mser	L- $\alpha$ -methylthreonine	Mthr
L- $\alpha$ -methylvaline	Mtrp	L- $\alpha$ -methyltyrosine	Mtyr
L- $\alpha$ -methylleucine	Mval Nnbhm	L-N-methylhomophenylalanine	Nmhpe
N-(N-(2,2-diphenylethyl) carbonylmethyl-glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbonylmethyl(1)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl ethylamino)cyclopropane	Nmbc		

The peptides of the present invention are preferably utilized in a linear form, although it will be appreciated that in cases where cyclization does not severely interfere with peptide characteristics, cyclic forms of the peptide can also be utilized.

- 5 Cyclic peptides can either be synthesized in a cyclic form or configured so as to assume a cyclic form under desired conditions (e.g., physiological conditions).

It will be appreciated that since one of the main obstacles in using short peptide fragments in therapy is their proteolytic degradation by stereospecific cellular proteases, the peptides of the present invention are preferably synthesized from D-  
10 isomers of natural amino acids [*i.e.*, inverso peptide analogues, Tjernberg (1997) J. Biol. Chem. 272:12601-5, Gazit (2002) Curr. Med. Chem. 9:1667-1675].

Additionally, the peptides of the present invention include retro, inverso, and retro-inverso analogues thereof. It will be appreciated that complete or extended partial retro-inverso analogues of hormones have generally been found to retain or  
15 enhance biological activity. Retro-inversion has also found application in the area of rational design of enzyme inhibitors (see U.S. Pat. No. 6,261,569).

As used herein a "retro peptide" refers to peptides that are made up of L-amino acid residues which are assembled in opposite direction to the native peptide sequence.

Retro-inverso modification of naturally occurring polypeptides involves the synthetic assembly of amino acids with  $\alpha$ -carbon stereochemistry opposite to that of the corresponding L-amino acids, *i.e.*, D- or D-allo-amino acids in inverse order to the native peptide sequence. A retro inverso analogue, thus, has reversed termini and reversed direction of peptide bonds, while essentially maintaining the topology of the side chains as in the native peptide sequence.

It will be appreciated that incorporation of any of the above-mentioned amino acid modifications including conserved changes in amino acid residues of the peptides of the present invention can be effected, as long as the angiogenic function (e.g., endothelial cell proliferation, migration, vascular sprouting, vascularization) of the peptides of the present invention is retained. To test this, any of the angiogenesis assays described hereinbelow and in the Examples section which follows can be effected.

The peptides of present invention can be biochemically synthesized, such as by using standard solid phase techniques. These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis. These methods are preferably utilized when the peptide is relatively short (*i.e.*, 10 kDa) and/or when it cannot be produced by recombinant techniques (*i.e.*, not encoded by a nucleic acid sequence) and therefore involves different chemistry.

Solid phase peptide synthesis procedures are well known in the art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Peptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

Synthetic peptides can be purified by preparative high performance liquid chromatography [Creighton T. (1983); Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via amino acid sequencing.

Recombinant techniques are preferably used when large amounts of the peptides are required. Such recombinant techniques are described by Bitter et al.,

(1987) *Methods in Enzymol.* 153:516-544, Studier et al. (1990) *Methods in Enzymol.* 185:60-89, Brisson et al. (1984) *Nature* 310:511-514, Takamatsu et al. (1987) *EMBO J.* 6:307-311, Coruzzi et al. (1984) *EMBO J.* 3:1671-1680 and Brogli et al., (1984) *Science* 224:838-843, Gurley et al. (1986) *Mol. Cell. Biol.* 6:559-565 and Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, NY, Section VIII, pp 421-463.

To produce a peptide of the present invention using recombinant technology, a polynucleotide encoding a peptide of the present invention (e.g., SEQ ID NO: 1, 3, 5, 7, 9 or 11) is ligated into a nucleic acid expression construct, which includes the polynucleotide sequence under the transcriptional control of a promoter sequence suitable for directing constitutive tissue specific or inducible transcription in the host cells, as further described hereinbelow.

Other than containing the necessary elements for the transcription and translation of the inserted coding sequence, the expression construct of the present invention can also include sequences engineered to enhance stability, production, purification, yield or toxicity of the expressed polypeptide. Such a fusion protein can be designed so that the fusion protein can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the heterologous protein. Where a cleavage site is engineered between the peptide moiety and the heterologous protein, the peptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that disrupts the cleavage site [e.g., see Booth et al. (1988) *Immunol. Lett.* 19:65-70; and Gardella et al., (1990) *J. Biol. Chem.* 265:15854-15859].

A variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the peptide coding sequence. These include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vector containing the peptide coding sequence; yeast transformed with recombinant yeast expression vectors containing the peptide coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the peptide coding sequence. Mammalian expression systems can also be used to express the peptides of the present invention. Bacterial systems are

preferably used to produce recombinant peptides, according to the present invention, thereby enabling a high production volume at low cost.

Other expression systems, such as insects and mammalian host cell systems, which are well known in the art, can also be used by the present invention.

5 In any case, transformed cells are cultured under effective conditions, which allow for the expression of high amounts of recombinant peptides. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce the recombinant peptides of the present invention. Such a medium typically includes an aqueous solution having  
10 assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen  
15 content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant proteins of the present invention may either remain within the recombinant cell; be secreted into the fermentation medium; be secreted into a space between two cellular  
20 membranes, such as the periplasmic space in *E. coli*; or be retained on the outer surface of a cell or viral membrane.

Following a certain time in culture, recovery of the recombinant protein is effected. The phrase "recovering the recombinant protein" refers to collecting the whole fermentation medium containing the protein and need not imply additional  
25 steps of separation or purification. Proteins of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential  
30 solubilization.

The peptides of the present invention are preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein in the diverse applications, described herein.

As mentioned hereinabove, the peptides of the present invention can be used to promote angiogenesis (*i.e.*, vascularization) in a tissue of a subject even under hypoxic conditions.

As used herein the term "subject" refers to a mammal, such as a canine, a feline, a bovine, a porcine, an equine. Preferably, the subject of the present invention is human.

The subject of this aspect of the present invention may suffer from an angiogenesis-dependent disease or disorder. Examples include, but are not limited to delayed wound-healing, delayed ulcer healing, reproduction associated disorders, arteriosclerosis, myocardial ischemia, peripheral ischemia, cerebral ischemia, retinopathy, remodeling disorder, von Hippel-Lindau syndrome, diabetes and hereditary hemorrhagic telangiectasia.

It will be appreciated that the peptides of the present invention can also be expressed from a nucleic acid construct administered to the subject employing any suitable mode of administration, described hereinabove (*i.e.*, in-vivo gene therapy). Alternatively, the nucleic acid construct is introduced into a suitable cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the individual (*i.e.*, ex-vivo gene therapy). However, to enable secretion of the peptides of the present invention the polynucleotides encoding thereof (e.g., SEQ ID NO: 1, 3, 5, 7, 9 or 11) preferably further include a polynucleotide sequence which encodes an in-frame signal peptide (e.g., the signal peptide of human VEGF-B Swiss-Prot/TrEMBL Accession VEGF\_HUMAN).

To enable cellular expression of the peptides of the present invention, the nucleic acid construct of the present invention further includes at least one cis acting regulatory element. As used herein, the phrase "cis acting regulatory element" refers to a polynucleotide sequence, preferably a promoter, which binds a trans acting regulator and regulates the transcription of a coding sequence located downstream thereto.

Any available promoter can be used by the present methodology. In a preferred embodiment of the present invention, the promoter utilized by the nucleic acid construct of the present invention is active in the specific cell population

transformed. Examples of cell type-specific and/or tissue-specific promoters include promoters, such as albumin that is liver specific [Pinkert *et al.*, (1987) *Genes Dev.* 1:268-277], lymphoid specific promoters [Calame *et al.*, (1988) *Adv. Immunol.* 43:235-275]; in particular promoters of T-cell receptors [Winoto *et al.*, (1989) *EMBO J.* 8:729-733] and immunoglobulins; [Banerji *et al.* (1983) *Cell* 33:729-740], neuron-specific promoters, such as the neurofilament promoter [Byrne *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477], pancreas-specific promoters [Edlunh *et al.* (1985) *Science* 230:912-916] or mammary gland-specific promoters, such as the milk whey promoter (U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). The nucleic acid construct of the present invention can further include an enhancer, which can be adjacent or distant to the promoter sequence and can function in up regulating the transcription therefrom.

The nucleic acid construct of the present methodology preferably further includes an appropriate selectable marker and/or an origin of replication. Preferably, the construct utilized is a shuttle vector, which can propagate both in *E. coli* (wherein the construct comprises an appropriate selectable marker and origin of replication) and be compatible for propagation in cells, or integration in a gene and a tissue of choice. The construct according to the present invention can be, for example, a plasmid, a bacmid, a phagemid, a cosmid, a phage, a virus or an artificial chromosome.

Currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral or non-viral constructs, such as adenovirus, lentivirus, Herpes simplex I virus, or adeno-associated virus (AAV) and lipid-based systems. Useful lipids for lipid-mediated transfer of the gene are, for example, DOTMA, DOPE, and DC-Chol [Tonkinson *et al.*, *Cancer Investigation*, 14(1): 54-65 (1996)]. The most preferred constructs for use in gene therapy are viruses, most preferably adenoviruses, AAV, lentiviruses, or retroviruses. A viral construct, such as a retroviral construct includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means, such as alternate splicing, nuclear RNA export, or post-translational modification of messenger. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and positive and negative strand primer binding sites appropriate to the virus used, unless it is already present in the viral construct. In addition, such a

construct typically includes a signal sequence for secretion of the peptide or antibody from a host cell in which it is placed. Preferably the signal sequence for this purpose is a mammalian signal sequence. Optionally, the construct may also include a signal that directs polyadenylation, as well as one or more restriction sites and a translation  
5 termination sequence. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof. Other vectors can be used that are non-viral, such as cationic lipids, polylysine, and dendrimers.

The peptides or the nucleic acid construct encoding same of the present  
10 invention can be provided to an individual *per se*, or as part of a pharmaceutical composition where one peptide or more is mixed with a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components,  
15 such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the peptide preparation or the nucleic acid construct encoding same, which is accountable for the biological effect.

20 Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases. One of the ingredients included in the  
25 pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter *et al.* (1979).

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient.  
30 Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, 5 transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer a preparation in a local rather than systemic 10 manner, for example, via injection of the preparation directly into a specific region of a patient's body.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or 15 lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. 20 Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers, such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the 25 formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, 30 suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in

particular, fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations, such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers, such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler, such as lactose, binders, such as starches, lubricants, such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base, such as lactose or starch.

The preparations described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents, such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acids esters, such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The preparation of the present invention may also be formulated in rectal compositions, such as suppositories or retention enemas, using, e.g., conventional suppository bases, such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro assays. For example, a dose can be formulated in animal models and such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human.

5 The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. [See e.g., Fingl, *et al.*, (1975) "The Pharmacological Basis of Therapeutics", Ch. 1 p.1].

10 Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

15 Compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

20 Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

30 Due to their selective binding to endothelial cells, the peptides of the present invention can be used to target agents fused thereto to ECs and thus can also be used for treating, *i.e.*, curing, preventing or substantially reducing symptoms of angiogenesis-dependent diseases which are characterized by hyper-vascularization.

For example, such fusions which include drugs can be used to inhibit tumor growth by destruction of the tumor vasculature.

Examples of drugs which can be included in such compositions include, but are not limited to, toxins, such as enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof [e.g., diphtheria toxin, exotoxin A chain of *Pseudomonas aeruginosa*, ricin A chain, abrin A chain, modeccin A chain,  $\alpha$ -sarcin, Aleurites fordii proteins, dianthin proteins, Phytolacca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes], radioisotopes (e.g.,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{90}\text{Y}$ ,  $^{212}\text{Bi}$  and  $^{198}\text{Re}$ ) and chemotherapeutic agents (e.g., alkylating agents, folic acid antagonists, anti-metabolites of nucleic acid metabolism, antibiotics, pyrimidine analogs, 5-fluorouracil, cisplatin, purine nucleosides, amines, amino acids, triazol nucleosides, or corticosteroids. Specific examples include, Adriamycin, Doxorubicin, 5-Fluorouracil, Cytosine arabinoside (*i.e.*, Ara-C), Cyclophosphamide, Thiotepa, Busulfan, Cytosin, Taxol, Toxotere, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan, and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors, such as tamoxifen and onapristone.

Fusions between the peptides of the present invention and drugs can be generated using a variety of bifunctional protein-coupling agents, such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bisazido compounds (such as bis-(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin fusion can be prepared as described in Vitetta *et al.*, Science, 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-

methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the peptide. See WO94/11026; U.S. Pat. No. 6,426,400; Laske, D. W., Youle, R. J., and Oldfield, E. H. (1997) Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumors. *Nature Medicine* 3:1362-1368.

A growing body of evidence indicates that angiogenesis is essential to the progression of cancer. In fact, the extent of neovascularity is strongly correlated with metastases in primary breast carcinoma, bladder cancer, prostate cancer, non-small cell lung cancer, cutaneous melanomas, and uterine cervix carcinoma [Ferrara, N., *Breast Cancer Research and Treatment* 36: 127-137 (1995)]. Thus, assessing the angiogenic phenotype of tumors will provide a strong indication to disease outcome. Other diseases or conditions which are characterized by hypervascularization or hypovascularization include, but are not limited to, retinal neovascularization, neovascularization in atherosclerotic plaques, hemangiomas, arthritis, and psoriasis, as well as the diseases described hereinabove. See Folkman, J. *New England J. of Med.* 333:1757-63 (1995).

Thus, the ability of the peptides of the present invention to bind specifically to the cell-surface of endothelial cells, suggests the use thereof as potent detectors of vascularization. This may be important for detecting the presence of, assessing predisposition to, or monitoring progression of an angiogenesis-dependent diseases.

Thus, the present invention also envisages a method of detecting a presence or an absence of endothelial cells in a biological sample.

The method is effected by incubating the biological sample with a peptide of the present invention capable of binding to the cell-surface of endothelial cells and detecting the peptide, to thereby detect the presence or the absence of endothelial cells in the biological sample.

The biological sample utilized for detection is preferably a tissue sample such as a biopsy specimen. Methods of obtaining tissue biopsies from mammals are well known in the art (see <http://www.healthatoz.com/healthatoz/Atoz/default.html>).

At least one peptide of the present invention is contacted with the biological sample under conditions suitable for complex formation (*i.e.*, buffer, temperature, incubation time etc.); suitable conditions are described in Example 1 of the Examples section.

Peptide-cell complexes within a biological sample can be detected via any one of several methods known in the art, which methods can employ biochemical and/or optical detection schemes.

To facilitate complex detection, the peptides of the present invention are highlighted preferably by a tag or an antibody. It will be appreciated that highlighting can be effected prior to, concomitant with or following complex formation, depending on the highlighting method. As used herein the term "tag" refers to a molecule, which exhibits a quantifiable activity or characteristic. A tag can be a fluorescent molecule including chemical fluorescers, such as fluorescein or polypeptide fluorescers, such as the green fluorescent protein (GFP) or related proteins ([www.clontech.com](http://www.clontech.com)). In such case, the tag can be quantified via its fluorescence, which is generated upon the application of a suitable excitatory light. Alternatively, a tag can be an epitope tag, a fairly unique polypeptide sequence to which a specific antibody can bind without substantially cross reacting with other cellular epitopes. Such epitope tags include a Myc tag, a Flag tag, a His tag, a leucine tag, an IgG tag, a streptavidin tag and the like.

It will be appreciated that the peptides of the present invention may also be used as potent detectors of endothelial cells *in vivo*. A designed peptide capable of binding endothelial cells, labeled non-radioactively or with a radio-isotope, as is well known in the art can be administered to an individual to diagnose the onset or presence of angiogenesis-dependent disease, discussed hereinabove. The binding of such a labeled peptide after administration to endothelial cells can be detected by *in vivo* imaging techniques known in the art.

It will be appreciated that such a detection method can also be utilized in an assay for uncovering potential drugs useful in inhibition or promotion of angiogenesis. For example, the present invention may be used for high throughput screening of test compounds (*i.e.*, putative angiogenic molecules). Typically, the peptides of the present invention are radiolabeled, to reduce assay volume. The peptides are allowed to bind endothelial cells prior to, concomitant with or following binding of the test compound. A competition assay is then effected by monitoring displacement of the label by a test compound [Han (1996) J. Am. Chem. Soc. 118:4506-7 and Esler (1996) Chem. 271:8545-8].

Once a putative angiogenic molecule is identified it is further evaluated using angiogenesis assays which are well known in the art. Examples include, but are not limited to, the chick chorioallantoic membrane, rabbit cornea assay, sponge implant models, matrigel and tumor models (see also the assays described in the Examples section which follows).

The peptides of the present invention can be included in a diagnostic or therapeutic kit. For example, the peptides can be packaged in a one or more containers with appropriate buffers and preservatives and used for diagnosis or for directing therapeutic treatment. Thus, the peptides, for example, can be each mixed in a single container or placed in individual containers. Preferably, the containers include a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials, such as glass or plastic.

In addition, other additives, such as stabilizers, buffers, blockers and the like may also be added.

The peptides of such kits can also be attached to a solid support, such as beads, array substrate (e.g., chips) and the like and used for diagnostic purposes.

Peptides included in kits or immobilized to substrates may be conjugated to a detectable label, such as described hereinabove.

The kit can also include instructions for determining if the tested subject is suffering from, or is at risk of developing, a condition, disorder, or disease associated with dysregulated angiogenesis.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

### **EXAMPLES**

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.* (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites *et al.* (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak *et al.*, "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

**EXAMPLE 1****SELECTION OF NOVEL POTENTIAL ANGIOGENESIS-INDUCING PHAGE  
DISPLAY PEPTIDES**

Novel peptides that potentially induce angiogenesis were identified by positive  
5 affinity selection (*i.e.*, biopanning) of a random phage display peptide library using  
human umbilical vein endothelial cells [HUVECs, ECs (the two abbreviations are  
used interchangeably throughout the document)], followed by Enzyme-Linked  
Immunosorbent Assay (ELISA) of positive phage clones to ECs.

***Materials and Experimental Methods***

10 ***Phage Display Peptide Library*** - The Random Phage Display Peptide Library  
employed in this study was purchased from New England Biolabs (NEB), Inc.  
(Beverly, MA, USA). The phage display library is based on a combinatorial library  
of random peptide 12-mers fused to a minor coat protein (pIII) of M13 phage. The  
displayed 12-mer peptides are expressed at the N-terminus of pIII. The library  
15 consists of about  $2.7 \times 10^9$  electroporated sequences amplified once to yield 20 copies  
of each sequence in 10  $\mu$ l of phage suspension.

The phage display peptide library was screened by five rounds of positive  
affinity selection (biopanning) using differentially-treated ECs: a) ECs without  
treatment (under normoxia), b) ECs following 3 hours of hypoxia treatment, and c)  
20 ECs following 24 hours of hypoxia treatment. Each positive selection was preceded  
by a negative selection using human Peripheral Blood Lymphocytes (PBLs). Each  
round of biopanning was effected by elution of the bound phage with 0.2 M glycine-  
HCl and incubation of the unbound phages on the second EC plate. This procedure  
was executed three times. Phages of the three elution steps were pooled for the  
25 second round of biopanning and so on. After the fifth round of biopanning, 40  
individual clones from each group of cells screened were isolated so that, in all, 120  
individual clones were obtained.

***Human Umbilical Vein Endothelial Cells (HUVECs)*** - HUVECs (ECs), were  
isolated by Collagenase digestion as previously described [Jaffe *et al.*, J. Clin. Invest.,  
30 52(11):2757-64, 1973]. ECs were cultured with M199 supplemented with 20 % FCS,  
 $10^4$  units of penicillin, 10 mg/ml of streptomycin sulfate, 10 mg/ml of neomycin  
sulfate (Biological Industries, Kibbutz Beit Haemek, Israel), 25  $\mu$ g/ml of EC growth

supplement (Biomedical Technologies, Inc., Stoughton, MA, USA), and 5 U/ml of Heparin (SIGMA, Rehovot, Israel). HUVECs were harvested with Trypsin (0.25 %), EDTA (0.05 %, Biological Industries, Kibbutz Beit Haemek, Israel) and incubated on 60 mm petri dishes coated with 1 % gelatin for 24 hours. Following incubation, cells were washed and incubated with M199 supplemented with 10 % FCS. ECs were subjected to four different treatments: a) no treatment, b) 3 hours of hypoxia, c) 6 hours of hypoxia, or d) 24 hours of hypoxia. Subsequently, monolayers were washed with PBS and dried overnight. Cells were rehydrated with PBS containing 5 % FCS and 0.1 % sodium azide and maintained at 4 °C until biopanning.

**Hypoxia treatment** - ECs were subjected to hypoxia for 3, 6, or 24 hours in a gas mixture containing 94 % N<sub>2</sub>, 5 % CO<sub>2</sub>, and 1 % O<sub>2</sub> in a hypoxia chamber (Billups-Rothenberg, Delmar, CA, USA).

**Screening of positive clones by ELISA** - The binding of positive clones from each group was re-evaluated by ELISA. For this purpose, ECs under normoxic conditions, ECs following 3, 6, or 24 hours of hypoxia, or human PBL (as controls) were plated at  $20 \times 10^3$  cells/well on 96 well plates. Plates were re-hydrated by overnight incubation at 4 °C in PBS supplemented with 3 % BSA, followed by three washes with PBS. Phage from each of the 120 clones isolated were dispersed on the ELISA plates at concentrations of  $10^{10}$ ,  $10^9$ , or  $10^8$  phage per well and incubated for two hours at room temperature. Plates were then washed three times with PBS containing 0.05 % Tween, followed by three washes with double distilled water (DDW). After washing, an anti-M13 HRP antibody (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK) at a 1:5,000 dilution was added and incubated for 2 hours at room temperature °C, following which the plates were washed 5 times in the presence of PBS and 0.05 % Tween-20. The HRP reaction was carried out using 100 µl of tetramethyl benzidine liquid substrate (DAKO TMB substrate chromogen, DAKO Corporation, Carpinteria, CA, USA) for a 15 minute-incubation following which the reaction was terminated by the addition of 50 µl of 1 M HCl. Plates were read at 450 nm in an ELISA reader (SLT 400ATC, SLT LAB Instruments, , Austria).

**Statistical and graphical methods** - Statistical analysis was effected by analysis of variance (ANOVA) with appropriate post-hoc tests, generally Dunnett's,

for a comparison to a control or Tukey-Kramer HSD for multiple comparisons. Results were considered statistically significant at  $P < 0.05$ .

### **Results**

#### ***EC-binding peptides were selected using phage display peptide library - A***

5 phage display peptide library was subjected to five rounds of positive affinity selections (biopanning) using ECs under physiological conditions (*i.e.*, normoxia) or following hypoxia. The second step of selection of peptide-presenting phage was effected by ELISA using ECs and lymphocyte-coated plates as controls. Fifteen different peptide-presenting phages at a concentration of  $10^9$  (Figure 1a) and  $10^{10}$  (Figure 1b) phages per well were screened by ELISA on four different EC preparations (ECs at normoxic conditions and ECs following 3, 6, and 24 hours of hypoxia). Figure 1a and Table 3 hereinbelow, illustrate selected peptides which exhibited statistically significant differences ( $p < 0.05$ ) between binding of NO phage (*i.e.*, unmodified M13 phage) and binding of certain peptide-presenting phages as  
15 determined using ANOVA analysis of  $10^9$  selected phage indicated

**Table 3**  
***Peptide-presenting phage***

<b><i>Peptide</i></b>	<b><i>Cells</i></b>
SP	EC
SP	H3
TR	EC
TR	H3
TR	H6
TR	H24
VL	EC
VL	H3
VL	H6
VL	H24
YR	EC
YR	H3
YR	H6
YR	H24

20 Table 3: Peptide-presenting phage selected from EC (ECs at normoxic conditions), H3 (ECs following 3 hours of hypoxia), H6 (ECs following 6 hours of hypoxia), and H24 (ECs following 24 hours of hypoxia).  $P < 0.05$ .

25 Similarly, statistically significant peptides which were selected using ANOVA analysis performed on  $10^{10}$  phages ( $p < 0.05$ ) are shown in Figure 1b.

Altogether, these findings demonstrate the identification of specific peptide-presenting phages which are capable of specifically binding ECs under either normoxia or hypoxia.

5

## **EXAMPLE 2**

### ***SELECTED PEPTIDE-PRESENTING PHAGES ARE CAPABLE OF INDUCING ANGIOGENESIS IN VITRO***

10

The ability of selected peptide-presenting phages to induce angiogenesis *in vitro* was evaluated by inducing EC proliferation, migration or sprouting of aortic rings.

#### ***Materials and Experimental Methods***

##### ***Identification of DNA sequences from selected peptide-presenting phages -***

15

DNA from all isolated selected clones was purified by incubation with iodide buffer and ethanol according to the manufacturer's instructions (NEB, Beverly, MA, USA). This rapid procedure produces template of sufficient purity for automated DNA sequencing with dye-labeled dideoxynucleotides. The 96 gIII (NEB) sequencing primer was utilized for automated DNA sequencing by the Sequencing Unit of Tel Aviv University, Tel Aviv, Israel.

20

***ECs and hypoxia treatment*** - ECs were isolated, cultured, and subjected to hypoxia treatments as described in Example 1, hereinabove.

25

30

***EC proliferation assay*** - ECs ( $40 \times 10^3$  cells/well) were seeded on 24-well plates coated with 1 % gelatin and were further cultured in the presence of M199 medium supplemented with 20 % FCS,  $10^4$  units of penicillin, 10 mg/ml of streptomycin sulfate, 10 mg/ml of neomycin sulfate (Biological Industries, Kibbutz Beit Haemek, Israel), 25  $\mu$ g/ml of endothelial cell growth supplement (Biomedical Technologies, Inc., Stoughton, MA, USA) and 5 U/ml of heparin (SIGMA, Rehovot, Israel). After 24 hours, cells were washed with serum-free medium (SFM) and incubated with SFM for another 24 hours at 37 °C. Subsequently,  $10^6$  selected phages were added per well and plates were incubated for another 24 hours. Abortive phages (those lacking a cloned peptide) were used as negative controls and were designated NO. Two  $\mu$ Ci/well of [ $^3$ H]-Thymidine (SIGMA, Rehovot, Israel) were added for the last 6 hours of incubation, following which the cells were fixed for 16 hours with 10

% TCA at 4 °C and washed with absolute ethanol. For cell lysis, NaOH (300 µl of 0.5 M per well) was added to the cells for a 15-minute incubation at 37 °C, following which cell lysates were transferred to scintillation vials containing 2 ml of scintillation liquid (Ultima Gold, Packard Bioscience, Meriden, CT, USA) and counted cpm/min in a β counter (Scintillation β Counter 1600 TR, Packard A Camberra Company, Meriden, CT, USA).

**EC migration assay** - EC migration was evaluated by the Chemicon QCM 96-well Migration Assay (Chemicon International, Temecula, CA, USA) according to the manufacturer's instructions. Briefly, the kit's migration chamber comprises an insert membrane with 8 µm pores and a feeder tray containing the peptides as chemoattractants. Cell migration was evaluated by placing the cells in the migration chamber and following their migration to the bottom of the membrane.

For the migration/chemoattractant assay, ECs from passage 3 were incubated for 24 hours on gelatin-coated plates in the presence of the M199 SFM. Following trypsinization,  $2 \times 10^4$  ECs were incubated for 5 hours in each of the 96 wells of the migration chamber. The peptide-presenting phages ( $10^5$  or  $10^6$ ) were added to the feeder tray to chemoattractant the cells without being in physical contact with the cells. Cells which reached the bottom of the membrane (*i.e.*, migratory cells) were dissociated from the membrane following the incubation with a cell detachment buffer. The migratory cells were subsequently lysed and detected by the molecular probe CyQuant GR dye which exerts green fluorescent enhancement when bound to cellular nucleic acid.

For activation migration assays,  $10^5$  or  $10^6$  peptide-presenting phages were incubated (for 5 hours) with ECs in the migration chamber. NO phages (unmodified M13 phages) served as negative control in both migration assays. Results were determined by a fluorescent ELISA reader at 480/520 nm (Fluostar BMG Lab Teck) and are presented in net values from which the control values were subtracted.

**Aortic ring formation assay** - Adventitia of human mammary or radial artery was stripped and cut into 1 mm long rings. The bottom of each well of a sterile 96-well plate was coated with 20 µg of fibronectin (Biological Industries, Kibbutz Beit Haemek, Israel) and the rings were positioned in the center of each well containing 150 µl of Dulbecco's modified Eagle's medium (DMEM, Biological Industries)

supplemented with 10 % FCS. For aortic ring formation,  $10^6$  peptide-presenting phages were added to each well and the plates were incubated for 7 days in the presence of 5 % CO<sub>2</sub> at 37 °C. Unmodified M13 phages (NO) were used as negative controls. Arterial rings were removed and the extent of cell proliferation was estimated using the XTT assay (Biological Industries) according to the manufacturer's instructions.

### ***Experimental Results***

***Characterization of identical EC-binding peptides from various EC-treated cells*** - DNA sequence analysis of the cloned regions of the positively selected peptide-presenting phages revealed the presence of identical clones among the various ECs. Moreover, some peptide-presenting phages (e.g., VL, LP, TR) were common in both cells grown under normoxia (EC) and cells grown under hypoxia (H24). Other peptide-presenting phages, e.g., YR or LT, were from ECs exposed to 3 or 24 hours of hypoxia, respectively. On the other hand, while SP was common to ECs exposed to 3 and 24 hours of hypoxia, others (e.g., ST, QF, NS), were from ECs grown under normoxia (Table 4, hereinbelow).

***Table 4***  
***Number of identical sequences obtained from positively-selected peptide-presenting phages***

<b><i>Peptide</i></b>	<b><i>EC</i></b>	<b><i>H3</i></b>	<b><i>H24</i></b>
VL	22	-	10
LP	2	29	9
TR	2	-	2
ST	2	-	-
QF	4	-	-
NS	2	-	-
SP	-	3	2
YR	-	2	-
LT	-	-	4
HR	-	-	3
HY	-	-	2
TP	-	-	-
NR	-	-	-
SA	-	-	-

Table 4: The number of identical clones present in positively-selected peptide-presenting phages from EC (ECs under normoxic conditions), H3 (ECs following 3 hours of hypoxia), or H24 (ECs following 24 hours of hypoxia).

**Peptide-presenting phages are capable of inducing EC proliferation and migration** - Six individual clones (VL, LT, QF, SP, YR, and TR) were tested using the Chemicon QCM 96-well Migration Assay for the capacity of the presented peptides to induce EC proliferation and migration. The DNA and protein sequences of the selected peptides are displayed in Table 5, hereinbelow.

**Table 5**  
**Sequences of selected peptides and nucleic acid encoding same**

PeptideID #	Nucleic acid sequence (SEQ ID)	Amino acid sequence (SEQ ID)
VL	GTTCCGTGGATGGAGCCGGCTTATCAGAGGTTTCTG (SEQ ID NO:1)	VPWMEPAYQRFL (SEQ ID NO:2)
LT	CTGCTTGCGGATACGACGCATCATAGGCCGTGGACT (SEQ ID NO:3)	LLADTTHRPWT (SEQ ID NO:4)
QF	CAGCCTTGTTGGAGCAGGCTTATTATAGTACGTTT (SEQ ID NO:5)	QPWLEQAYYSTF (SEQ ID NO:6)
SP	TCTGCGCATGGGACGTCTACTGGTGTCCGTGGCCG (SEQ ID NO:7)	SAHGTSTGVPWP (SEQ ID NO:8)
YR	TATCCGCATATTGATTGCTTGGTCATTGGCGGCGG (SEQ ID NO:9)	YPHIDSLGHWR (SEQ ID NO:10)
TR	ACTTTGCCGTGGCTGGAGAGTCTTATTGGCGTCCT (SEQ ID NO:11)	TLPWLEESYWRP (SEQ ID NO:12)

Table 5: Presented are the amino acid sequences of the selected peptides and the nucleic acid sequences encoding same.

As is shown in Figure 2, all six selected peptide-presenting phages induced (at a concentration of  $10^6$ /well) increased proliferation of ECs as compared with the unmodified, empty, phages (NO).

**The selected peptide-presenting phages are capable of inducing EC migration** - The ability of the peptide-presenting phages to induce EC migration of activated ECs was tested by placing the peptide-presenting phages with the ECs in migration chambers. Two of the tested peptide-presenting phages (QF and LT) induced migration of the activated ECs at a concentration of  $10^5$  (Figure 3a) or  $10^6$  (Figure 3b) phages per well. On the other hand, placement of the peptide-presenting phages at two different concentrations on the feeder tray revealed the ability of YR (of the six peptide-presenting phages) to induce migration as chemoattractants at  $10^5$  phages per well (Figure 4a), and the ability of QF, SP, TR and LT to induce migration as chemoattractants at a concentration of  $10^6$  phages per well (Figure 4b).

**Aortic ring sprouting by peptide presenting phages** - Aortic rings were tested for sprouting in the presence of peptide-presenting phages. Figure 5 demonstrates

proliferation of cells originating from the aortic rings, induced by peptide presenting phages. ANOVA analysis comparing the six peptide-presenting phages indicated an overall clear difference in the proliferation of cells derived from the aortic rings ( $P = 0.0003$ ). In addition, post-hoc tests indicated statistically significant differences  
 5 between the VL peptide-presenting phage and the S24 empty phage control (*i.e.*, NO).

These results demonstrate that the peptide-presenting phages of the present invention are capable of inducing EC migration and proliferation, *i.e.*, are capable of inducing angiogenesis *in vitro*.

### 10 **EXAMPLE 3**

#### **SYNTHETIC PEPTIDES ARE CAPABLE OF INDUCING ANGIOGENESIS IN VITRO UNDER NORMOXIC CONDITIONS**

Peptides corresponding to the selected peptide-presenting phages were synthesized and their potential to induce angiogenesis *in vitro* was evaluated, as  
 15 follows.

##### ***Materials and Experimental Methods***

**Peptide synthesis** - Peptides were synthesized by SynPep (Dublin, CA, USA). HPLC purity analysis demonstrated that the purity of each synthetic peptide was higher than 97 %. Peptide QF was dissolved in 50 % water / 50 % acetonitrile. All  
 20 other peptides were dissolved in water.

**Fluorescein labeling of synthetic peptides** - Fluorescein Isothiocyanate (FITC, Pierce, Rockford, IL) is an amino-reactive probe that reacts in an alkaline environment with primary amines to form a stable fluorescent derivative. 12.5  $\mu$ l of FITC (10 mg/ml) were added per 1 mg of peptide diluted in 0.5 M bicarbonate buffer  
 25 (pH 9.5) and agitated in the dark for 2 hours. 0.1 ml of 1.5 M hydroxylamine was then added per 1 ml of reaction mixture and agitated for an additional 30 minutes at room temperature. Unbound FITC was removed by dialysis in the presence of PBS.

**Peptide binding to ECs** - ECs were cultured in M199 supplemented with 10 % FCS. For peptide binding assays, the cells were trypsinized and  $10^5$  cells were  
 30 suspended in PBS supplemented with 5 % FCS and 0.1 % sodium azide. Cells were incubated for 2 hours (on ice, in the dark) in the presence of 1-6  $\mu$ g of FITC-labeled peptides, following which the stained cells were washed twice with PBS. Samples

were analyzed by FACS (FACScan, Becton Dickinson, San Jose, CA, USA). For control,  $0.5 \times 10^6$  of PBLs were utilized.

***Proliferation of ECs in the presence of synthetic peptides*** - ECs were incubated in the presence of EBM-2 medium containing supplements (Cambrex BioWhittaker Cell Biology Products, Walkersville, USA). Cells were passaged every 3 days by harvesting cells with 0.25 % Trypsin / 0.05 % (Biological Industries) and re-plating the cells at a concentration of  $10^4$  cells per a 25 cm<sup>2</sup> flask. ECs from passage 3 were used for proliferation experiments.

ECs ( $12 \times 10^3$  cells/well) were seeded on 24-well plates in EBM-2 medium containing supplements. Following a 24-hours incubation, cells were subjected to 24 hours of starvation in supplements-free medium (SFM). Synthetic peptides (SP, LT, TR, and VL) were each added at concentrations of 0.05, 0.1, 1, 10, or 100 ng/ml for 24 hours. For a proliferation assay, 2  $\mu$ Ci/well of [<sup>3</sup>H]-Thymidine (SIGMA, Rehovot, Israel) were added for a 6-hour incubation, following which the plates were washed 3 times with PBS. For cell lysis, the plates were incubated for 15 minutes at 37 °C with 300  $\mu$ l/well of 0.5 M NaOH. Subsequently, cell lysates were transferred to scintillation vials containing 2 ml of scintillation liquid (Ultima Gold, Packard Bioscience, Meriden, CT, USA) and counted (cpm/min) in a  $\beta$  counter (Scintillation  $\beta$  Counter 1600 TR, Packard A Cambera Company, Meriden, CT, USA).

***Proliferation of dermal microvascular endothelial cells (MVECs) in the presence of synthetic peptides*** - MVECs were incubated with synthetic peptides (LT, SP, and YR) as described above for HUVECs (ECs), except that the MVECs were seeded in EBM-MV medium containing supplements (Cambrex BioWhittaker Cell Biology Products) and MVECs from passage 4 (rather than 3) were used for proliferation experiments.

***Migration assays in the presence of synthetic peptides*** - EC migration was evaluated as described in Example 2, hereinabove. Following trypsinization, ECs ( $25 \times 10^3$ ) were incubated in migration chambers. For the chemoattractant migration assay, synthetic peptides were added to the feeder tray at 5, 10, 20, and 50 ng/ml and incubated with the cells for 5 or 15 hours. For migration activation assays, synthetic peptides at 0.1, 1, and 10 ng/ml were incubated with the cells in the migration chamber for 5 or 15 hours.

MVEC migration was evaluated as described above except that MVECs from passage 3 were incubated in EBM-MV SFM. Synthetic peptides were added at 10 ng/ml to the feeder tray for chemoattractant migration assay as well as for activation of migration assays. The presence of migratory cells was detected using the fluorescent ELISA reader at 480/520 nm (Fluostar BMG Lab Teck) as described in Example 2, hereinabove.

***Sprouting of aortic rings by synthetic peptides*** - Human mammary or radial artery was prepared in a 96-well plate as described in Example 2, hereinabove. Peptides were added in increasing concentrations (1, 10, 100, and 1,000 ng/ml) to each well containing the aortic ring. Plates were incubated at 37 °C in the presence of 5 % CO<sub>2</sub> for 7 days. Arterial rings were removed and cell proliferation was assessed using the XTT assay (Biological Industries) according to the manufacturer's instructions.

***Tube formation assay*** - ECs from passage 3 or MVECs from passage 4 were harvested with trypsin and incubated for 24 hours in SFM. Twenty-four - well plates were pre-coated with 250 µl of Cultrex Basement Membrane Extract with reduced growth factors (R&D Systems, Minneapolis, MN, USA). Five hundred microliters of medium containing 10<sup>6</sup> cells were transferred to the coated wells. Synthetic peptides, FGF, YR, QF, or VL were added to ECs, and VEGF, YR, QF, or VL were added to MVECs at 10 ng/ml. Plates were incubated for 24 hours at 37 °C in the presence of 5 % CO<sub>2</sub>. HUVECs and MVECs were photographed under a light microscope at 20 hours and 8 hours, respectively.

***Real Time PCR*** - MVECs from passage 3 were incubated for 24 hours in EBM-MV supplement-free medium (starving media). Following starvation, 1 ng/ml of synthetic peptides LT, QF, SP, TR, YR, and VL and 10 ng/ml of VEGF were added to the plates. Following 1.5 or 6 hours of incubation, total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad CA, USA) and 0.8 µg of the extracted RNA was used as a template for reverse transcription (Invitrogen) using random primers (SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad CA, USA) according to the manufacturer's instructions. Resulting cDNAs were subjected to real time PCR amplification using the ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Oligonucleotide primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA) according the translated region of VEGF-A (Accession No:NM\_003376), VEGF-C (Accession No:NM\_005429), KDR (Accession No:NM\_002253), FLT-1 (Accession No:NM\_002019), HIF- $\alpha$  (Accession No:HSU22431), and GAPDH (Accession No:NM\_002046) and are listed in Table 6, hereinbelow. Briefly, a reaction mixture of 20  $\mu$ l consisting of DDW, oligonucleotide primers (500 nM), cDNA (3  $\mu$ l), and SYBR Green PCR master kit (Applied Biosystems, Foster City, CA, USA) was subjected to an amplification program of 15 seconds at 95  $^{\circ}$ C, and 60 seconds at 60  $^{\circ}$ C for 40 cycles. Results were analyzed using the Sequence Detector Software Version 1 (Applied Biosystems).

**Table 6**  
***Oligonucleotide primers for amplification of selected genes cDNA***

<b><i>Gene name</i></b>	<b><i>Sense primer (SEQ ID)</i></b>	<b><i>Anti-sense primer (SEQ ID)</i></b>
VEGF-A	CTACCTCCACCATGCCAAGTG (SEQ ID NO:15)	TGCGCTGATAGACATCCATGA (SEQ ID NO:16)
VEGF-C	TTCCTGCCGATGCATGTCTA (SEQ ID NO:17)	TGTTCGCTGCCTGACACTGT (SEQ ID NO:18)
KDR	TCAGGCAGCTCACAGTCCTAGAG (SEQ ID NO:19)	ACTTGTCGTCTGATTCTCCAGGTT (SEQ ID NO:20)
FLT-1	TCAGCGCATGGCAATAATAGA (SEQ ID NO:21)	ACCAAGGTGCTAGCCATCTTATTC (SEQ ID NO:22)
HIF- $\alpha$	AGTGTACCCTAACTAGCCGAGGAA (SEQ ID NO:23)	GCCTGTGCAGTGCAATACCTT (SEQ ID NO:24)
GAPDH	GTCGGAGTCAACGGATTTGG (SEQ ID NO:25)	GGCAACAATATCCACTTTACCAGAGT (SEQ ID NO:26)

***Statistical and graphical methods*** - See Example 1, hereinabove.

### ***Experimental Results***

***Synthetic peptides bind ECs in vitro*** - Six synthetic peptides LT, QF, SP, TR, VL, and YR (displayed in Table 5) were synthesized in order to evaluate their ability to induce angiogenesis *in vitro* and *in vivo*. Specific binding of the above-described synthetic peptides to ECs was tested. The peptides were FITC-labeled and binding to ECs was analyzed by FACS. As is shown in Figures 6a-i and Figure 7, the synthetic peptides bound specifically to ECs but not to lymphocytes (PBLs). Increasing concentrations of peptides (Figures 6c-i, red lines) resulted in increased binding to ECs (94-96 % binding) relative to a lower concentration (Figures 6c-i, green lines).

***Synthetic peptides are capable of inducing proliferation under normoxia -***

Synthetic peptides were assayed for their effect on proliferation of HUVECs under normoxic conditions. ECs were seeded on 24 well plates in SFM for 24 hours and then synthetic peptides were added at increasing concentrations for an additional 24 hours. A significant dose-dependent increase in [<sup>3</sup>H]-Thymidine uptake was observed in ECs incubated with the LT, SP, TR, and VL peptides (Figure 8a). Peptides LT, TR, and SP, at 10 ng/ml each, induced the highest proliferative response, leading to 1.7, 1.8, and 1.6-fold increases, respectively. On the other hand, VL, at 1 ng/ml, induced the highest proliferative response leading to a 1.8 fold increase.

A significant increase in [<sup>3</sup>H]-Thymidine uptake was also demonstrated for MVECs incubated with LT, SP or YR (Figure 8b). The increase was in a dose dependent manner with the highest proliferation response in a concentration of 1 ng/ml for all three peptides. At this concentration, LT YR and SP increased MVECs proliferation response by 1.8, 1.7 and 1.4 fold, respectively (Figure 8b).

***Cells migration by synthetic peptides*** – To test the effect of the synthetic peptides on EC cell migration, synthetic peptides at concentrations of 5, 10, 20, and 50 ng/ml were added to the feeder tray. A dose-dependent induced migration was observed for LT (Figure 9a) and SP (Figure 9b). The effect appears to reach a plateau at high concentrations, which would be predicted based on pharmacokinetics. The exact pharmacologic profile of this attraction requires further study. A smaller effect of induced migration was noted for VL and TR when used as chemoattractants (Figure 9c).

***The synthetic peptides are capable of activating migration of EC cells*** – To further test the effectiveness of the synthetic peptides in activating migration, the synthetic peptides were incubated with the ECs in the migration chambers. Figure 10 illustrates that each of these peptides, within a 5 hours span, induces statistically significantly more ECs migration than control ECs without the peptide present. By 15 hours, however, the migration of cells has been reduced, so that no statistical difference is seen between any peptide-treated cells and control epithelial cells (Figure 10).

MVECs were also shown to migrate due to synthetic peptides induction. The experiment performed with the test peptides demonstrated their effectiveness as chemoattractants to induce migration of MVECs in a dose dependent manner. Figure

11a illustrates that each of the test peptides, within a 5 hours span, induced more cell migration than control endothelial cells without the peptide present (results are in net values from which the control values were subtracted). At 10 ng/ml, LT, QF, YR SP, TR and VL induced a 3.5, 2.4, 2.4, 2, 2, and 2 – fold increase in MVECs migration respectively.

MVECs were also directly activated by the synthetic peptides. TR, SP, QF, and YR were all shown to induce MVECs migration (by 2.6, 2.1, 2.3 and 1.8 folds, respectively), at 1 ng/ml (Figure 11b).

**Aortic rings sprouting by synthetic peptides** - As described before for the peptide-presenting phages, aortic rings induced sprouting was evaluated by addition to the cultured aortic rings purified synthetic peptides at different concentrations. Four peptides (QF, YR, LT, and VL) were compared for their ability to induce cell proliferation in aortic rings. Clear differences between the peptides (after correction for control optical density) were observed at the noted peptide concentrations (Figure 12).

**Tube Formation** - ECs and MVECs were incubated on matrigel in the presence of peptides and array formation was analyzed. Peptides (YR, QF and VL) added to MVECs at concentration of 10 ng/ml, resulted in a significant increase in tube formation (Figures 13c-e) as compared to untreated cells (Figure 13a). This increase was similar to the effect of VEGF on these cells (Figure 13b). The same effect of increased tube formation was induced by these peptides when added to ECs (Figure 13h-j) as compared to untreated cells (Figure 13f). This increase was similar to the effect of FGF on ECs (Figure 13g).

As described for the peptide-presenting phages, the synthetic peptides produced, could induce *in vitro* angiogenic effects in ECs. In all angiogenic effects tested, at least one (while in most cases more) of the synthetic peptides tested showed significant effect over control. These results indicate that these peptides may be also capable of *in vivo* angiogenesis.

**Peptides effect on gene expression in MVECs** - The effect of the synthetic peptides on the expression in MVECs of selected genes (VEGF-A, VEGF-C, KDR, FLT-1 and HIF-1 $\alpha$ ) related to the VEGF pathway (a major pathway that participated in angiogenic process) was tested. Synthetic peptides were added at 1 ng/ml for 1.5

and 6 hours. After incubation with peptide, RNA was extracted from MVECs and Real Time PCR was performed. Gene expression was calculated as peptide / control (cells without peptide treatment) ratio.

The different peptides exhibited varying effects on the expression of the genes tested as demonstrated in Figures 14a-e and summarized in Table 7, hereinbelow.

**Table 7**  
***The effect of the different peptides on gene expression***

<i>Gene</i>	<i>Synthetic peptide</i>	<i>Expression after 1.5 hours</i>	<i>Synthetic peptide</i>	<i>Expression after 6 hours</i>
VEGF-A (Figure 14a)	QF, TR, YR, VL, LT, SP	+	VL QF, TR, YR	+ -
VEGF-C (Figure 14b)	QF, TR, VL, LT, SP	+	QF, TR, VL, LT, SP	+
FLT-1 (Figure 14c)	QF, TR, YR, VL, LT, SP	+	QF, YR	-
KDR (Figure 14d)	QF, SP, YR LT	+ -	TR, VL YR	+ -
HIF (Figure 14e)	QF, TR, VL LT, YR	+ -	TR, VL LT, QF	+ -

Table 7: The increased (+) or non-increased (-) gene expression is presented for the noted genes as a result of incubation with the noted synthetic peptides.

As is shown in Table 7 hereinabove, all 6 peptides tested were shown to induce the expression of some of the genes tested. These results may lead to the molecular mechanism by which these peptides induce angiogenesis.

#### **EXAMPLE 4**

##### ***THE EFFECT OF HYPOXIA ON IN VITRO INDUCED ANGIOGENESIS BY SYNTHETIC PEPTIDES***

The ability of selected peptides to induce angiogenesis was evaluated by induction of cell binding, proliferation, migration and tube formation assays under hypoxic condition.

##### ***Materials and Experimental Methods***

***Peptides synthesis and Fluorescein labeling*** - See Example 3, hereinabove.

***ECs and hypoxia conditions*** - ECs were isolated, cultured, and subjected to hypoxia conditions as described in Example 1, hereinabove.

***FACS analysis of peptide binding to ECs with and without hypoxia treatment*** - ECs were exposed to hypoxia conditions and then prepared for FACS analysis as described in Example 3, hereinabove. Cells were stained with 6 µg of SP or LT labeled peptide. Samples were analyzed by Fluorescence Activated Cell Sorter (FACScan Beckton Dickinson, CA, USA).

***Synthetic peptides induced proliferation or migration after and under hypoxia treatment*** - Cell proliferation or migration assays were performed as described in Example 3, hereinabove, except that cells were divided to 3 groups: control cells, cells after exposure to hypoxia conditions or cells proliferating under hypoxia conditions. For cell proliferation assays, ECs were incubated with QF, LT and SP and MVECs were incubated with LT, SP, and YR. For cell migration assays, ECs were incubated with EBM-2 and MVECs were incubated with EBM-MV media.

***Tube formation assay after and under hypoxia treatment*** - Tube formation assay was performed as described in Example 3, hereinabove, except that the cells were divided to 3 groups: control cells, cells after exposure to hypoxia conditions, and cells on matrigel basement that form tubes under hypoxia treatment.

***Statistical and graphical methods*** – was performed as described in Example 1, hereinabove.

### ***Experimental Results***

***Synthetic peptides bind to ECs after hypoxia treatment*** - Peptide binding assays were performed on ECs grown under normoxia or ECs following hypoxia. As is shown in Figures 15 and 16a-b, while LT and SP exhibited increased binding to ECs exposed to hypoxia, the other peptides (*i.e.*, QF, TR, VL and YR) exhibited similar intensity of binding to ECs under both conditions.

***Synthetic peptides induced proliferation under and following hypoxia treatment*** - The effect of synthetic peptides (QF, LT and SP) on proliferation of ECs was tested under and following hypoxia treatment as compared to control. ECs were seeded on 24 well plates in serum free media for 24 hours, following which synthetic peptides were added to cells in various concentrations for additional 24 hours.

Figures 17a-b demonstrate a significant dose-dependent increase in [<sup>3</sup>H]-Thymidine uptake in ECs incubated with the LT and SP peptides under and following hypoxia. As is shown in Figure 17a, while LT (at a concentration of 10 ng/ml)

resulted in a 3.5-fold increase of cell proliferation after hypoxia, LT at 1 ng/ml resulted in a 1.7-fold increase in cell proliferation under hypoxia. Similarly, SP (at a concentration of 10 ng/ml) increased cell proliferation after hypoxia in 2 fold (Figure 17b). On the other hand, QF did not show increase in proliferation of endothelial cells after or under hypoxia treatment (Figure 17c).

The effect of these synthetic peptides (QF, LT and SP) on the proliferation of MVECs under the same conditions was also tested. Figures 17d-e demonstrate a significant dose-dependent increase in [<sup>3</sup>H]-Thymidine uptake in MVECs which were incubated with the peptides LT and SP after and under hypoxia. LT increased 1.7 fold MVECs proliferation after hypoxia at 1 ng/ml and at 10 ng/ml under hypoxia conditions (Figure 17d). SP increased MVECs proliferation after hypoxia in 1.5 fold at 10 ng/ml and 1.9 fold under hypoxia conditions at 1 ng/ml (Figure 17e). QF did not show increase proliferation of MVECs under hypoxic conditions compared to normoxic conditions (Figure 17f).

Table 8, hereinbelow, summarizes the set of experiments testing the effect of the synthetic peptides of the present invention on HUVEC or MVEC cell proliferation under normoxia, following hypoxia or under hypoxia.

**Table 8**  
***The effect of synthetic peptides on cell proliferation***

	<b>PROLIFERATION</b>					
	<i>Normal</i>		<i>After hypoxia</i>		<i>Under hypoxia</i>	
	<b>HUVEC</b>	<b>MVEC</b>	<b>HUVEC</b>	<b>MVEC</b>	<b>HUVEC</b>	<b>MVEC</b>
<b>YR</b>	++	++	-	-	+	+
<b>LT</b>	-	++	++	++	++	++
<b>SP</b>	-	-	++	++	++	++
<b>QF</b>	+	+	+	-	-	-
<b>TR</b>	++	++	-	-	-	-
<b>VL</b>	++	-	-	-	+	+
<b>FGF</b>	++	++	++	+	+	+

Table 9, hereinbelow, summarizes the set of experiments testing the effect of the synthetic peptides of the present invention on HUVEC or MVEC cell migration.

**Table 9*****The effect of synthetic peptides on cell migration***

<b>MIGRATION</b>				
	<b>Normal conditions</b>		<b>Under hypoxia</b>	
	<b>HUVEC</b>	<b>MVEC</b>	<b>HUVEC</b>	<b>MVEC</b>
None	-	-	-	-
YR	+	+	+	+
LT	+++	+++	-	-
SP	-	+	+	++
QF	++	++	+	++
TR	++	+	+	++
VL	+	+	++	+
FGF	++	++	+	+

5

***Tube formation assay after and under hypoxia treatment*** - The effect of synthetic peptides of the present invention on tube formation of ECs and MVECs was tested by their incubation on matrigel in the presence of LT, SP and QF peptides.

Addition of 10 ng/ml peptides QF under normoxic conditions, similar to the effect of VEGF or bFGF, resulted in a significant increase in tube formation in comparison to untreated ECs (Figure 13d and i). SP and LT under normoxic conditions did not induce tube formation (Data not shown). Peptide SP however, was effective only in tube formation under hypoxia conditions (Figure 18a-e).

Table 10, hereinbelow, summarizes the set of experiments testing the effect of the synthetic peptides of the present invention on tube formation.

**Table 10*****The effect of synthetic peptides on tube formation***

<b>TUBE FORMATION</b>						
	<b>Normal conditions</b>		<b>After hypoxia</b>		<b>Under hypoxia</b>	
	<b>HUVEC</b>	<b>MVEC</b>	<b>HUVEC</b>	<b>MVEC</b>	<b>HUVEC</b>	<b>MVEC</b>
None	-	-	-	-	-	-
YR	++	++	-	-	++	++
LT	-	-	-	-	-	-
SP	-	-	-	-	++	++
QF	++	++	+	+	-	-
TR	-	-	-	-	-	-
VL	++	++	-	-	-	-
FGF	++	++	++	++	-	-

20

**EXAMPLE 5****PEPTIDE- INDUCED IN VIVO ANGIOGENESIS**

The synthetic peptides of the present invention were used to induce *in vivo* angiogenesis in a mouse ear model and rat or mice ischemic hind-limb models.

5       ***Materials and Experimental Methods***

***In vivo angiogenesis in a mouse-ear model*** - Ear angiogenesis studies were a modification of an approach described previously (Pettersson, 2000). Synthetic peptides in a concentration of 1, 10 and 20 µg/15 µl per mouse were injected sub-cutaneously into the ears of nude mice and Balb/C mice. Contralateral ears were  
10 injected only with PBS. Digital photographs were obtained 2, 4, 6, and 20 days after injection. Two days after peptide inoculation, angiogenic effect of peptides could be observed.

***Histological sections*** - Histological sections of the mouse-ears injected with the angiogenic peptides were performed by fixing tissues in 4 % buffered formalin.  
15 Sections were embedded in paraffin blocks sectioned in 4 µm thick layers and stained with hematoxolin-eosin.

***Rat ischemic hind-limb model and laser-Doppler imager analysis*** - A rat ischemic hind limb model was used for evaluation of the *in vivo* potential of angiogenesis induced by the selected synthetic peptides. Ischemia was created in the  
20 rat left hind limb by ligation the femoral artery. The right hind limb served as a control. A day after the operation each of the peptides was injected into two sites close to the ligation and one site distal to the ligation. Each rat was treated with each of the peptides in a total amount of 600 µg.

      The blood flow was measured using a Laser Doppler Blood Flow analyzer  
25 (MoorLDI, Moor Instrument, Wilmington, Delaware) at 2, 6, 9 and 13 days after peptides injections. The average perfusion of each limb was computed and blood flow was expressed as the ischemic (left) / control (right) blood flow ratio.

***Mouse ischemic hind limb model*** - Ischemia was created in the mouse left hind limb by ligation of the femoral artery. The right hind limb served as control. A  
30 day after the operation each of the peptides was injected into one site close to the ligation and one site distal to the ligation. Each mouse was treated with each of the peptides in a total amount of 10 µg.

**Physiological observations** – Ischemic mice were evaluated for their ability to climb a ladder on day 1, 4, 7, and 10-post operation. The scoring system was as follows: 1 - walk and climb; 2 - walk and climb with some difficulty; 3 - walk and cannot climb the ladder; 4 - walk with difficulties and cannot climb the ladder.

5 **Blood perfusion in ischemic mice** - The percent of blood perfusion was measured using a Laser Doppler Imager (PeriMed, Sweden) at 14 and 19 days after peptides injections. The average percent perfusion of each limb was computed and expressed as the ischemic (left) / control (right) blood perfusion ratio.

10 **Statistical and graphical methods** – were performed as described in Example 1, hereinabove.

### **Experimental Results**

15 **In vivo angiogenesis in a mouse-ear model** - Injection of the synthetic peptides or VEGF into the ears of nude mice and Balb/C mice resulted in increased number of blood vessels in the ears of mice injected with 10 µg of LT, YR, QF and SP (Figures 19b-e) or VEGF (Figure 19a). Histological examination of stained sections of the ears revealed an increase in the number of blood vessels and the appearance of neo-vascularizations in peptide injected ears (Figures 20a-b, Table 11, hereinbelow).

20 **Table 11**  
**Blood vessels induced by peptide injection**

<i>Peptide</i>	<i>Concentration</i>	<i>Days after inoculation</i>	<i>Number of blood vessels</i>
FGF	3 ng/ear	5	11
control	-	5	7
VEGF	10 ng/ear	5	17
control	-	5	10
LT	10 µg/ear	5	18
control	-	5	13
SP	10 µg/ear	5	15
control	-	5	9
YR	10 µg/ear	5	11
control	-	5	12
TR	10 µg/ear	5	15
control	-	5	9
QF	10 µg/ear	5	21
control	-	5	15
VL	10 µg/ear	5	14
control	-	5	12

Table 12, hereinbelow, summarizes the data obtained from a set of experiments testing the effect of the peptides of the present invention on *in vivo* ear angiogenesis.

**Table 12**  
***In vivo ear angiogenesis induced by AngioPeptides***

	<i>No. of mice</i>	<i>PBS Injected</i>	<i>Peptide / PBS</i>
<b>PBS</b>	10	11.1	0.8
<b>LT</b>	10	11.3	3
<b>SP</b>	10	10	1
<b>YR</b>	8	12.1	7.6
<b>TR</b>	10	9.5	5.5
<b>VL</b>	10	9.8	5.6
<b>QF</b>	10	12.2	0.9
<b>FGF</b>	10	11.42	2.08

Table 12: Synthetic peptides or PBS were injected to mice ears as described under Materials and Experimental Methods and the number of blood vessels were counted in PBS injected or the peptide injected ear. Peptide/PBS = the ratio between the No. of blood vessels in the peptide-injected ear and the No. of blood vessels in the PBS-injected ear.

***Laser-Doppler analysis in a rat ischemic hind-limb model*** - The blood flow of ischemic hind limb was measured after 600 µg peptide injection using a Laser Doppler Blood Flow analyzer (MoorLDI, Moor Instrument) at 4 time points (at days 2, 6, 9 and 13). The percent of median flux of the operated leg / control leg of rats treated with peptides was calculated for each peptide injected. Treatment of rats with the peptides QF and YR showed 112.5 and 108.2 percent increase of median flux of the ischemic leg / control leg, respectively (Figure 21).

Physiological evaluation of the ability of the ischemic mice to climb a ladder were followed on day 1, 4, 7, and 10-post operation and the results are summarized in Table 13, hereinbelow.

**Table 13**  
***Physiological evaluation of ischemic mice following peptide injection***

Injection	Day 1	Day 4	Day 7	Day 10
PBS	3.07	3	2	2
FGF	2.57	2	1	1
YR	2.75	2.5	1.4	1
TR	3.05	2.5	1.6	1.4
QF	2.83	2	1.6	1.2
LT	3.07	2.25	1.6	1.2
VL	2.75	2	1.6	1.4
SP	2.85	2.25	1.75	1.6

Table 13: Physiological score of mice with hind limb ischemia as determined by the ability to climb a ladder. Shown are the mean scores of 10 mice in each group. The scoring system was as following: 1 - walk and climb; 2 - walk and climb with some difficulty; 3 - walk and cannot climb the ladder; 4 - walk with difficulties and cannot climb the ladder.

5

The results presented in Table 13, hereinabove, demonstrate the ability of the peptides of the present invention to prevent at least some of the physiological difficulties present in ischemic mice (e.g., climbing a ladder).

***Injection of the synthetic peptides of the present invention increases blood***

10 ***perfusion in ischemic mice*** – To further test the potential of the synthetic peptides of the present invention to induce angiogenesis *in vivo*, the percent of blood perfusion was measured in ischemic mice using a Laser Doppler Imager (PeriMed, Sweden) at 14 and 19 days following peptides injections. The average percent perfusion of each limb was computed and expressed as the ischemic (left) / control (right) blood  
15 perfusion ratio. Significant differences were observed between the peptides ( $P = 0.0102$ ). As is shown in Table 14, hereinbelow, a significant increase in the percent of blood perfusion ratio was observed in mice injected with QF and YR.

***Table 14***

20 ***The blood perfusion ratio (in percentages) of the ischemic vs. control limbs***

<b><i>Level</i></b>	<b><i>Number</i></b>	<b><i>Mean</i></b>	<b><i>Std Dev</i></b>	<b><i>Std Err Mean</i></b>
<b>FGF</b>	4	93.438	8.3423	4.171
<b>LT</b>	4	78.275	11.0320	5.516
<b>PBS</b>	9	82.436	7.2285	2.410
<b>QF</b>	4	112.222	22.4931	11.247
<b>SP</b>	8	84.478	15.2471	5.391
<b>TR</b>	8	86.006	16.4668	5.822
<b>VL</b>	8	95.560	12.2757	4.340
<b>YR</b>	4	104.349	22.0348	11.017

Altogether, these results strongly suggest the use of the synthetic peptides of the present invention, and especially, the QF and YR as angiogenic, anti-ischemic  
25 agents.

**EXAMPLE 6*****A CONSERVED SEQUENCE MOTIF (SEQ ID NO: 13) SUPPORTS THE ANGIOGENIC FUNCTION ATTRIBUTED TO THE PEPTIDES OF THE PRESENT INVENTION***

5           Sequence analysis of the isolated peptides revealed a conserved amino acid sequence (SEQ ID NO: 27 or 32) which is shared by 3-4 of the peptides VL; QF, TR and possibly YR (see Figures 22a-c).

          This sequence was found by the eMOTIF scan software (Biochemistry, Stanford University, <http://dna.stanford.edu/emotif/emotif-scan.html>) to be shared  
10   with mouse vascular endothelial growth factor B precursor (Swiss-Prot Accession: VEGF\_MOUSE), which has a very similar human homologue. The following peptide sequences YR (shared); LT and SP may belong to a different group. Interestingly these two groups of peptide were isolated under two different test conditions, while the first (VL, QF and TR) were isolated under normoxic conditions  
15   the second groups of peptides (YR, LT and SP) were selected under hypoxic conditions, suggesting that these two groups bind to different cellular determinants or with different affinities.

          It is appreciated that certain features of the invention, which are, for clarity,  
20   described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

25           Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications  
30   mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this

application shall not be construed as an admission that such reference is available as prior art to the present invention.

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